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PRINCIPAL INVESTIGATOR: **Mulu Geletu, PhD**

CONTRACTING ORGANIZATION: **Queen's University at Kingston**
Kingtonston, K7L 3N5
Canada

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6. AUTHOR(S)
Mulu Geletu, PhD (Principal Investigator)

geletu@queensu.ca

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Queen's University at Kingston
Kingston K7L 3N5, Canada

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13. SUPPLEMENTARY NOTES

14. ABSTRACT : Stat3 is activated by cytokine receptors as well as receptor and non-receptor tyrosine kinases. Activation of Stat3 has been demonstrated in breast and other cancers, while a constitutively active form of Stat3, Stat3C, is able to transform cultured cells, further pointing to an etiologic role for Stat3 in these tumors. However, the exact mechanism of its activation as well as the role of Stat3 in these tumors, remain to be determined. This study focuses on the identification of the upstream activators of Stat3 in confluent cells as well as the role of Stat3 in this setting. A quantitative RT-PCR array experiment using mouse breast epithelial HC11 cells demonstrated a dramatic increase in the levels of secreted IL6 family cytokines, while knockdown experiments demonstrated that this increase is required for Stat3 activation. We also demonstrated that density causes a dramatic increase in levels and activity of the Rac GTPase, due to inhibition of proteasomal degradation. Furthermore, we demonstrated that expression of mutationally activated Rac^{V12} leads to Stat3 activation through IL6 secretion, and this is required for cell motility, indicating that the gp130/Stat3 axis represents an essential effector for the regulation of key cellular functions. Examination of the role of Stat3 in confluent cultures showed that Stat3 inhibition leads to a dramatic activation of the p53 anti-oncogene. Interestingly, our results also showed that Stat3 inhibition in normal breast epithelial cells induced the p53 target, $p21^{CIP/WAF}$ but only at lower densities, consistent with previous results showing that $p21^{CIP/WAF}$ is associated with p53-mediated growth arrest.

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Stat3, ErBb2, Breast cancer

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Introduction

The signal transducer and activator of transcription-3 (Stat3) is activated by receptor tyrosine kinases cytokine receptors and non-receptor tyrosine kinases. Following ligand stimulation, Stat3 is activated through phosphorylation at tyr-705 and migrates to the nucleus where it activates the transcription of genes involved in cell growth and survival[1,2]. Persistent activation of Stat3 has been demonstrated in breast and other cancers, while a constitutively active form of Stat3, Stat3C, is able to transform cultured cells[3], further pointing to an etiologic role for Stat3 in these tumors. However, the exact mechanism of its activation as well as the role of Stat3 in these tumors and, hence the consequences of its inhibition remain to be determined.

Unlike tissue culture cells, cells in a tumor have extensive opportunities for adhesion to their neighbors in a three-dimensional structure, therefore in the study of cellular functions it is important to take into account the effect of surrounding cells. In fact, we recently demonstrated that engagement of the cell-cell adhesion proteins, ***E-cadherin*** or ***cadherin-11*** can lead to a dramatic activation of ***Stat3***[4]***. This was due to a striking upregulation of Rac1, a GTPase of the Rho family and known Stat3 activator, and followed by a potent downregulation of the p53 antioncogene and apoptosis inhibition. Most importantly, the cadherin-mediated, Stat3 activation was independent from a number of tyrosine kinases which are often activated in a variety of cancers, such as the Src family, IGF1-R and Fer, as well as the EGFR family. Therefore, this novel pathway of Stat3 activation that is triggered by specific cadherin engagement, could be a promising target for the treatment of breast cancers where ***inhibitors*** of ***tyrosine kinases known to be hyperactive in breast cancer***, such as the ErbB2 inhibitor ***Herceptin, would be ineffective***.

Body

Specific Aim 1. Examination of the upstream activators of Stat3 in confluent cells: Nature of the kinase(s) controlling Stat3 activity following cadherin engagement.

Our first goal was to identify receptor or non-receptor phosphoproteins (pR's) activated following cell to cell adhesion using a proteomics approach.

IL6-family cytokines are upregulated following cadherin engagement: Before embarking on a detailed proteomics analysis, we decided to first explore the possibility that the density-mediated, Stat3 activity increase might be due to secreted factors. A careful examination of Stat3 activity revealed that cell to cell adhesion does indeed trigger the expression of autocrine factors **(Fig. 1A, lane 2 *vs* 1).** To explore the nature of the cytokines that may be produced, we conducted a quantitative RT-PCR array for 86 cytokines, comparing sparsely growing, breast epithelial cells to cells grown as dense cultures. The results revealed a dramatic increase in mRNA levels of IL6 family cytokines, known to act through the common gp130 subunit, shared by a number of Stat3 activating cytokines, such as IL6, LIF, Ct1 and IL27. gp130 knockdown showed that these cytokines are actually ***required*** for the Stat3 activation observed in confluent cultures **(Fig. 1C upper panel)** (published in *Molecular Cancer Research*[4]). The focus of our efforts was then to identify additional players in this pathway.

Mutationally activated Rac activates Stat3 through IL6 induction: It was previously demonstrated that following HGF stimulation, Rac is degraded through the proteasome[5,6]. Our published results definitively demonstrated that cell density causes a dramatic increase in Rac protein and activity levels, which is due to inhibition of proteasomal degradation. Furthermore, pharmacologic inhibition of Rac and Cdc42, and genetic knockdown experiments demonstrated that Rac is required for Stat3 activation following cadherin engagement[4]*.

A corollary to the observation that Rac is required for the density-mediated, Stat3 activation, is that activated Rac expression lead to Stat3 activation. This was found to be indeed the case. Expression of Rac^{V12} or Rac^{L61} led to a Stat3 activity increase, at all cell densities **(Fig. 1D, lane 1-3).** Interestingly, although previous data demonstrated that constitutive activation of Rac, as well as binding of effectors which might be acting as ubiquitin E3 ligases, induce the degradation of Rac[27], our results show that Rac^{V12} or Rac^{L61} protein levels are increased dramatically with cell density, which demonstrates that cell density, can overcome the degradative effect of activation. A similar mechanism could hold true for Cdc42, which mirrored Rac levels and stability increases with cell density.

We next examined whether mutationally activated Rac^{V12} can induce IL6 expression, in a manner similar to cRac activation through cadherin engagement. To this effect, we conducted a quantitative RT-PCR array experiment for HC11 cells expressing Rac^{V12} *vs* the parental HC11, both grown to low densities, to eliminate cadherin-dependent effects. The results revealed an increase in mRNA levels of two cytokines of the IL6 family, IL6 (18-fold) and Leukemia inhibitory factor (LIF) (10-fold), known Stat3 activators. To further demonstrate the requirement for IL6 family cytokines for the Rac^{V12}-mediated, Stat3 activation, the levels of gp130 were reduced through stable expression of a specific shRNA, using a retroviral vector. As shown in **Fig. 1D**, gp130 knockdown reduced Stat3-ptyr705 levels in Rac^{V12}-expressing cells, at all densities examined **(lanes 1–3 vs. 4–6),** pointing to the possibility that the two IL6 family cytokines play an important role in the activation of Stat3 by Rac^{V12}. The upregulation of IL6 family cytokines was required for cell migration and proliferation induced by Rac^{V12}, as shown by gp130 knockdown experiments **(Fig. 2),** thus demonstrating that the gp130/Stat3 axis represents an essential effector of activated Rac for the regulation of key cellular functions.

An interesting spinoff directly derived from this work was the following:

Cell to cell adhesion inhibits Erk activation by IL6. Results from a number of labs indicated that, besides Stat3, IL6 stimulation results in activation of Erk1/2 (Erk)[7]. However, cadherin engagement and the concomitant IL6 increase ***does not activate Erk***[8,9]. To solve this apparent paradox, we examined the ability of IL6, or conditioned medium, to activate Erk in confluent cultures. HC11 cells were grown to 60% or 100% confluence, serum-starved and following IL6 stimulation, cell extracts were probed for Erk or Stat3-ptyr705. As shown in **Fig. 1B**, at a confluence of 60%, IL6 addition caused a dramatic increase in both Erk and Stat3 (~10x), in agreement with previous results[7]. As expected, cell density *per se* caused an increase in Stat3-ptyr705 levels **(Fig. 1B, top panel, lanes 1 *vs* 3),** and IL6 caused a further activation **(lane 4).** However, IL6 did ***not*** bring about an increase in Erk levels in densely growing cultures **(Fig. 1B, *middle*, lane 4 *vs* 2).** The above findings indicate that IL6 is unable to activate Erk in cells grown to high densities, thus revealing a profound effect of confluence on the response of HC11 cells to IL6 addition. In line with this conclusion, although Rac^{V12} activated Erk at low cell densities, it was unable to do so in dense cultures.

These results were published in *Experimental Cell Research*[10].

Since the qRT-PCR analysis and the downregulation of the gp130, common subunit showed that the IL6 family cytokines are responsible for the cadherin-dependent, Stat3 activation, we decided to postpone the proteomics analysis experiments for the moment. We also decided to use HC11 cells rather than MCF10A, because the latter present difficulties in genetic manipulation.

Specific Aim 2: Examination of the role of Stat3 in confluent cultures: Effect of Stat3 upon p53

Stat3 inhibition upregulates $p21^{CIP/WAF}$ but only at lower densities:

Our earlier data revealed a profound difference in the reaction of normal cells to Stat3 inhibition; Stat3 downregulation brought about growth arrest to subconfluent cultures, while confluent cells succumbed to apoptosis[11*]. The latter points to the possibility that the dramatic Stat3 activation by cell-cell adhesion may represent an attempt by the cell to overcome apoptotic death[8,12,13]. Further examination indicated that Stat3 inhibition with CPA7 increases p53 levels at all cell densities (**Fig. 3A, middle panel & 3B Bottom panel**), consistent with independent findings that Stat3 downregulates the p53 promotor by direct binding[14], and this could be abrogated by Stat3C expression with a lentiviral vector[3]. Interestingly, our results also showed that Stat3 inhibition in MCF-10A cells induced the p53 target, $p21^{CIP/WAF}$ but only at lower densities (**Fig 3A, bottom panel**). This is consistent with previous results showing that $p21^{CIP/WAF}$ is associated with p53-mediated growth arrest[15,16]. Whether Stat3 inhibition at high densities induces p53 targets previously associated with p53-mediated apoptosis, such as the *PUMA* and *NOXA*, BH3-only members of the Bcl-2 family (reviewed in[17]), will now be examined by Western blotting. These findings will be extended to demonstrate the effect of Stat3 inhibition using siRNA[13*,8*], the compound S3I-201[18], or a panel of peptidomimetics we developed[11*] which block the Stat3-SH2 domain and were found to be the strongest and most specific Stat3 inhibitors in a broad range of cultured cells (reviewed in[19,20]).

Role of caveolin-1: In our attempt to decipher the interrelationship between Stat3 and p53, we discovered another potential player: Caveolin 1 (cav1), a 22 KDa membrane protein is the major protein responsible for the organization and maintenance of caveolae microdomains[21,22]. Cav1 recruits many receptor and non-receptor tyrosine kinases and through binding to its scaffolding-domain, cav1 sequesters the kinases in

an inactive form, thereby preventing their involvement in signaling pathways[22]. Previous results showed that cav1 can activate p53[23]. Since Stat3 downregulates p53, we examined whether cav1 upregulates p53 through Stat3 inactivation. Our results to date indicated that cav1 overexpression does indeed lead to Stat3 downregulation, and this pathway will now be investigated further. These preliminary data were presented at the AACR meeting (2010, see below).

Our work led to two interesting spinoffs:

1. Stat3 is required for intercellular, gap junctional communication: Our observation that Stat3 activity is increased by cell confluence led us to examine the effect of Stat3 upon gap junctional, intercellular communication (GJIC), which is dependent upon cadherin engagement. The results turned out to be very interesting:

Neoplastic transformation by oncogenes such as activated Src is known to suppress gap junctional, intercellular communication (GJIC). One of the Src effector pathways leading to GJIC suppression and transformation is the Ras/Raf/Mek/Erk, so that inhibition of this pathway in vSrc-transformed cells restores GJIC. Stat3 is part of a distinct Src downstream effector pathway required for neoplasia, therefore we decided to examine its role in Src-mediated GJIC suppression. Testing of a number of breast cancer lines known to have high Src activity levels[8] revealed the absence of gap junctions, consistent with the known ability of Src to suppress GJIC. Unfortunately, the normal human breast epithelial line, MCF-10A and the HC11 mouse breast epithelial line have low GJIC, possibly due to their requirement for growth factors, known GJIC suppressors. For this reason, we resorted to the T51B, rat liver epithelial line which has extensive GJIC that can be suppressed by activated Src. Stat3 was downregulated in T51B-Src cells through treatment with the CPA7, Stat3 inhibitor, or through infection with a retroviral vector expressing a Stat3-specific shRNA. GJIC was examined by electroporating the fluorescent dye, Lucifer yellow, into cells grown on two co-planar electrodes of electrically conductive, optically transparent, indium-tin oxide, followed by observation of the migration of the dye to the adjacent, nonelectroporated cells under fluorescence illumination, using a novel technique we developed[24,25]. The results demonstrate that, contrary to inhibition of the Ras pathway, Stat3 inhibition in cells expressing activated Src does ***not*** restore GJIC. On the contrary, Stat3 inhibition in normal cells with ***high*** GJIC levels eliminated junctional permeability. Therefore Stat3, although it is generally growth promoting and in an activated form can act as an oncogene, its function is actually required for the maintenance of junctional permeability.

This is the first instance where an oncogene is actually ***required*** for gap junctional communication. This might be related to Stat3's ability to promote survival. These results were published in the Journal *DNA and Cell Biology*, with one of the figure placed on the cover of this issue.

2. Cell density increases the levels of GAPDH and α-tubulin, commonly used as "housekeeping" genes: In all of the above experiments where protein levels must be carefully quantitated, it is important to employ a gene product whose levels are not affected by cell density, as a control for protein loading. In our search for a suitable marker, we examined the effect of cell density upon the levels of a number of commonly used housekeeping gene products. The results showed that the levels of GAPDH and α-tubulin increased gradually with cell density starting at 10% confluence and plateauing at ~90%. Hsp90 and β-actin levels on the other hand did not change with cell density, from 10% confluence up to 5 days after confluence, indicating that these proteins may be appropriate controls. This is the first report on the effect of density of cultured cells upon the levels of certain commonly used, housekeeping gene products. These results were published in the *Journal of Immunological Methods*[26].

Specific Aim 3. Examination of the incidence of Stat3 activation, in conjunction with Rac1 and p53 levels in primary tumors, and correlation with the type of tumor, resistance to Herceptin, disease stage and outcome.

The very first hurdle before doing an extensive tissue microarray analysis (TMA), is to test the antibodies and the conditions of staining. A number of different antibodies were tested, and the following ones performed best: pan Stat3-Cell Signaling, #9132. pY705Stat3-Cell Signaling, #9131. p21- Cell Signaling, #2946. MDM-2- Sigma, # M-4308.

We initially prepared and stained a number of tumors manually by Immunohistochemistry. However, the Department of Pathology recently acquired an apparatus for automated staining. This approach provides

more robust staining conditions, and the department is in the process of setting up a new imaging system (ARIOL) for scoring of the arrays and quantitating the results of staining intensity using algorithms specific for nuclear, cytoplasmic or membranous staining. Therefore, the staining will be repeated with this advanced system. Moreover, we are introducing additional specificity controls for staining required for this integrated imaging approach.

Our findings that an increase in IL6 family cytokines is responsible for the cadherin-mediated, Stat3 activation indicated that IL6 could also be an important marker. Therefore, we will examine IL6 levels in TMA's and correlate with Stat3-ptyr705 and the other markers.

This project is ongoing as planned in collaboration with Dr. Bruce Elliott's lab. Developments of biomarkers and the identification of key signaling molecules in the pathway are in progress. We anticipate that data from this part of the project will be generated in the next year of the program.

Key Research Accomplishments

- Cell density induces the secretion of IL-6, a key activator of Stat3
- Cell density increases Rac/Cdc42 levels, through suppression of proteasomal degradation.
- Cell to cell adhesion inhibits Erk activation by IL6
- Stat3 inhibition with CPA7 increases p53 expression at all cell densities.
- At low cell density Stat3 inhibition causes an increase in $p21^{CIP/WAF}$ levels, concomitant with an inhibition of cell division rate.
- Stat3 inhibition in normal epithelial cells suppresses gap junctional, intercellular communication, indicating that Stat3 is required for junctional permeability.
- Contrary to inhibition of the Ras pathway, Stat3 inhibition in Src- transformed, epithelial cells does not restore GJIC.

Reportable Outcomes

Papers published

1.--Geletu, M., Chaize, C., Arulanandam, R., Vultur, A., Kowolik, C., Anagnostopoulou, A., Jove, R. and Raptis, L. (2009). Stat3 activity is required for gap junctional permeability in normal epithelial cells and fibroblasts. ***DNA and Cell Biology***, 28:319-27.

A Figure from this paper was placed on the cover.

2.--Arulanandam, R., Geletu, M., Feracci, H. and Raptis, L. (2010). Rac^{V12} requires gp130 for Stat3 activation, cell proliferation and migration. ***Experimental Cell Research*** **316**, 875-886).

3.--Greer S, Honeywell R, Geletu M, Arulanandam R and Raptis L. (2010). Cell density effects on the levels of housekeeping gene products. ***Journal of Immunological Methods*** **355**, 76-79.

4. Arulanandam, R, Geletu M and Raptis L. (2010) The Simian Virus 40 Large Tumor antigen requires Src for full neoplastic transformation. ***Anticancer Research***, 30:47-54.

5.--Raptis, L, Arulanandam, R., Vultur, A., Geletu, M., Chevalier, S. and Feracci, H. (2009) Beyond structure, to survival: activation of Stat3 by cadherin engagement. ***Biochemistry and Cell Biology*** **87**, 835-843

Abstract with oral presentation

Mulu Geletu, Reva Mohan, Rozanne Arulanandam, Adina Vultur, and Leda Raptis. 2010. Reciprocal regulation of Stat3 and the caveolae protein, cav-1**.** Annual resident and postdoctoral fellow research day February 18, Department of Pathology, Queen's University, Kingston, Ontario, Canada.

Abstracts with poster presentation

Mulu Geletu, Reva Mohan, Rozanne Arulanandam, Adina Vultur, and Leda Raptis. 2010. Reciprocal regulation of Stat3 and the caveolae protein, cav-1**.** 101th annual meeting of the American Association for Cancer Research, April 17-21, Washington DC, USA.

Chaize C, Geletu M, Arulanandam R, Firth K and Raptis L. Stat3 inhibition eliminates gap junctional communication in lung carcinoma cells. 48th annual meeting, the American Society for Cell Biology, San Francisco, CA.

Rozanne Arulanandam, Mulu Geletu, Adina Vultur, and Leda Raptis. 2009. The Simian Virus 40 Large Tumor antigen requires Src for full neoplastic transformation. 100th annual meeting of the American association for Cancer research, Denver, Colorado.

D'Abreo C, Arulanandam R, Geletu M and Raptis L. 2010. Activated Src increases total Rac levels and requires Rac and IL6 for full neoplastic transformation. 101th annual meeting of the American Association for Cancer research, April 17-21, Washington, DC.

Arulanandam, R., Geletu, M., Vultur, A., Cao, J., Larue, L., Feracci, H. and Raptis, L. 2010. Cadherin-cadherin engagement promotes cell survival via Rac/Cdc42 and Stat3. 101th annual meeting of the American association for Cancer research, April 17-21, Washington, DC.

Conclusions

Our finding of Stat3 activation by cadherin engagement has exposed Stat3 as a central determinant of the balance between cell proliferation and apoptosis. Our results demonstrate that cells grown as dense cultures have elevated mRNA levels of a number of cytokines including the IL-6 family that activate Stat3 through the common gp130 subunit. In addition, IL6 family cytokines also play an important role in the activation of Stat3 by Rac^{V12}. This pathway is required for cell proliferation and migration that is the gp130/Stat3 axis represents an essential effector of activated Rac for the regulation of key cellular functions. We have brought to light a novel pathway leading from E-cadherin engagement to Rac/Cdc42 and Stat3 activation and p53 downregulation, which is a key for the decision between these two opposing processes. Most importantly, this pathway could be a promising target for the treatment of cancers where inhibitors of tyrosine kinases, such as Herceptin, are ineffective.

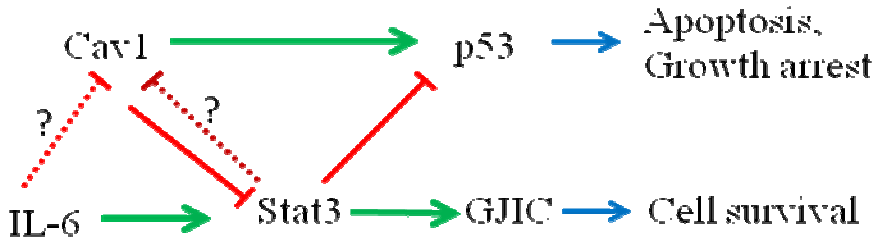
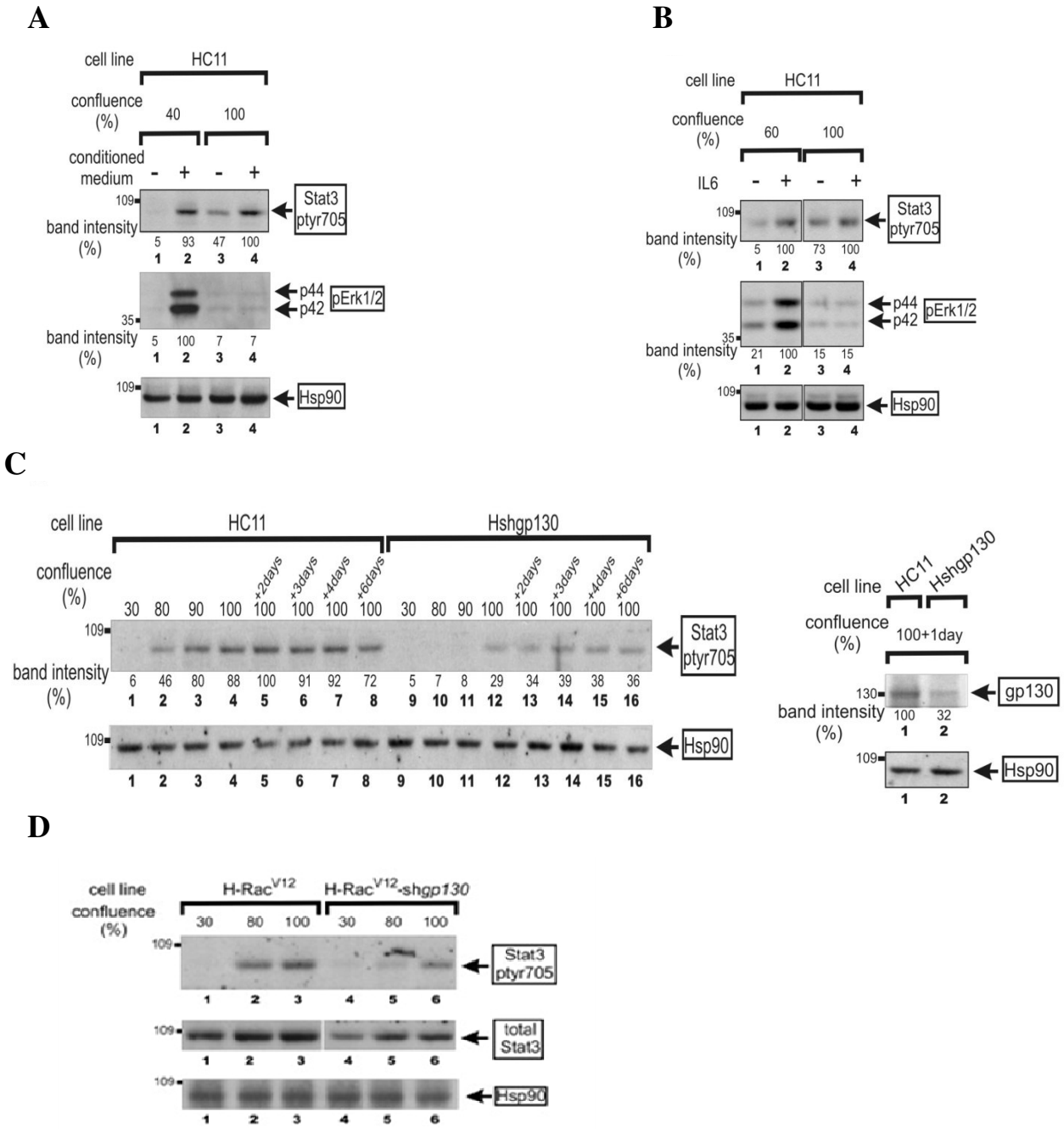
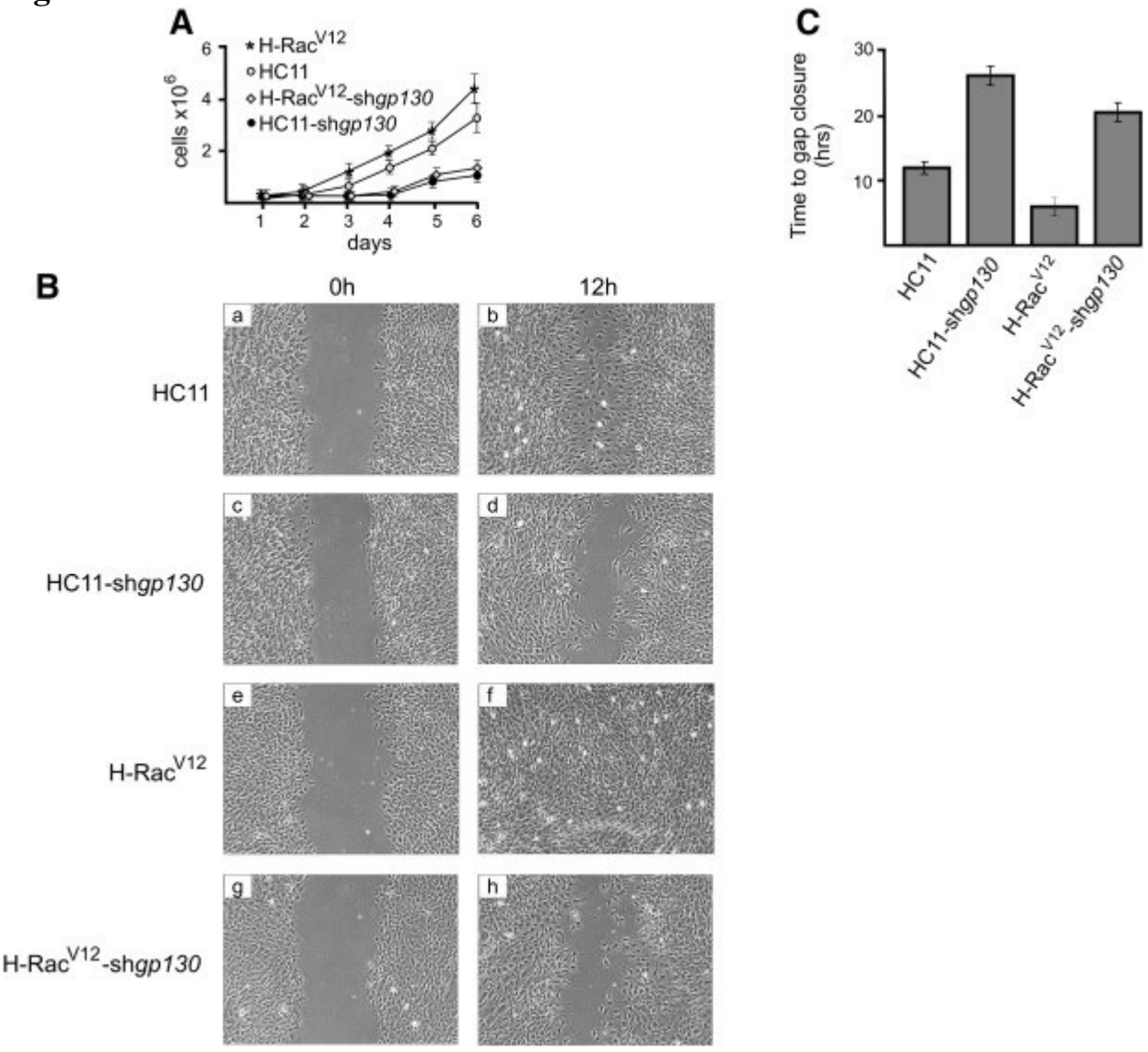


Figure 1.



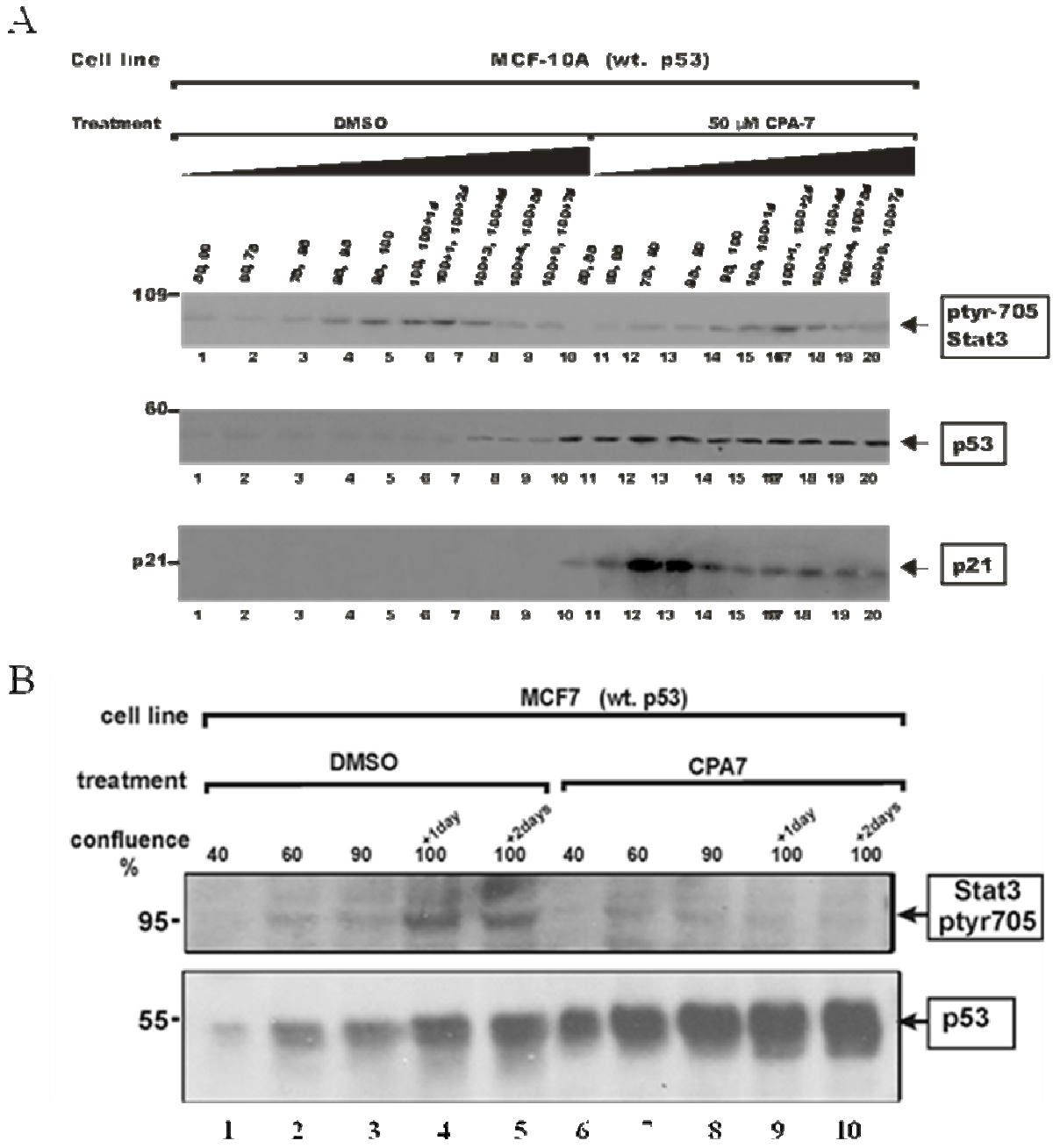
Cell to cell adhesion triggers cytokine gene expression. A: Confluence induces autocrine secretion of Stat3-activity factors. Conditioned medium from HC11 cells grown to 100% confluence was added for 15 min to HC11 cells grown to 40% (lane 2) or 100% (lane 4) confluence. At this time detergent cell extracts were resolved by gel electrophoresis and blots were cut into strips which were probed for Stat3-ptyr705, p-Erk1/2 or Hsp90 as indicated. Numbers under the lanes refer to Stat3-ptyr705 or p-Erk1/2 band intensities obtained through quantitation by fluorimager analysis, with the highest value taken as 100%. **B: IL6 activates Stat3 but not Erk at high densities.** IL6 was added for 15 min to HC11 cells grown to 40% (lane 2) or 100% (lane 4) confluence and cell extracts probed for Stat3-ptyr705, p-Erk1/2 or Hsp90 as indicated. Numbers under the lanes refer to Stat3-ptyr705 or p-Erk1/2 band intensities obtained through quantitation by fluorimager analysis, with the highest value taken as 100%. **C left pannel: gp130 downregulation reduces the density-mediated Stat3 activation.** HC11 cells before (lanes 1-8) or after (lanes 9-16) expression of shRNA for gp130 were grown to different densities and lysates probed for Stat3-ptyr705or Hsp90(bottom), as indicated. Numbers under the lanes refer to Stat3-ptyr705 or gp130 band intensities obtained through quantitation by fluorimager analysis, with the highest value taken as 100%. **C right pannel:** lysates from HC11cells, before (lane 1) or after (lane 2) knockdown of gp130 were probed for gp130 or Hsp90 as a loading control, as indicated.
D: gp130 is required for Rac^{V12}-mediated, Stat3 activation: Lysates from H-RacV12 cells before (lanes 1–3) or after (lanes 4–6) expression of a gp130-specific, shRNA were probed for Stat3-ptyr705, total Stat3 or Hsp90 as a loading control, as indicated. (from *Experimental Cell Research*[11])

Figure 2.



Rac-induced cell migration and proliferation require gp130. A: Cell proliferation: HC11, H-sh*gp130,* H-Rac^{V12}, or H-Rac^{V12}-sh*gp130* cells were grown in Petri dishes in 10% serum and cell numbers obtained over several days, as indicated. Values represent averages of 3 independent experiments. **B:** Cell migration: HC11, HC11-sh*gp130*, H-Rac^{V12} or H-Rac^{V12}-sh*gp130* cells were cultured to confluence before a scratch was made through the monolayer using a plastic pipette tip. Cells were photographed at 0 (panels a, c, e, g) or 24 h (panels b, d, f, h) after 12 h of culturing in 0.5% fetal calf serum. **C:** Quantitation of the time (h) required by the different cell lines for gap junction closure. The time taken for gap junction closure was determined by microscopic observation. Numbers represent averages of 3 independent experiments. (*Experimental Cell Research*[11])

Figure 3.



Stat3 inhibition by CPA7 upregulates p53 and p21$^{CIP/WAF}$ levels at low cell densities. A MCF-10A and **B** MCF7 cells grown to different densities as indicated, were treated with either the DMSO carrier (Fig.A lanes 1-10 &Fig.B lanes 1-5) or 50μM CPA7 Fig.A (lanes 11-20 & Fig.B lanes 6-10) for 24 hrs. Blots of detergent extracts of total protein were subsequently probed for **A:** Stat3-ptyr705**,** p53 or p21$^{CIP/WAF}$ and **B**: Stat3-ptyr705 or p53. The indicated degrees of confluence correspond to confluence before and after treatment. Numbers at the left refer to molecular-weight markers. Arrows point to the position of Stat3-ptyr705, p53 or p21$^{CIP/WAF}$. Note the dramatic increase in p53 levels with CPA7 treatment (Fig. 3 A & B lanes 11-20 & lanes 6-10), while p21$^{CIP/WAF}$ is increased at lower densities only (Fig.3 A lanes 11-14).

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Appendices: None